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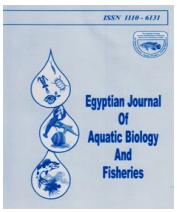

Artificial Spawning and Gametogenic development of the blacklip pearl oyster, *Pinctada margaritifera* Linnaeus, 1758 (Bivalvia: Mytilidae) from Mabahiss Bay, the Red Sea

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ABSTRACT

The study focused on understanding the reproductive biology of *Pinctada margaritifera*, commonly known as the black-lipped pearl oyster, which holds significant economic importance due to its production of Tahitian cultured pearls. Research conducted in Mabahiss Bay, the Red Sea, Egypt throughout 2022 provided crucial insights into the reproductive cycle of *P. margaritifera*. The species exhibits protandric hermaphroditism, transitioning from male to female during its growth. Shell sizes inversely correlated with sex distribution, with a sex ratio of 1.05: 1.0: 0.1 for females, males, and undifferentiated individuals, respectively. The study identified a distinct spawning phase from June to October, peaking in July and August. Analyses of gonad index fluctuations in females and males provided additional understanding of reproductive dynamics. Among the methods tested to stimulate spawning, hormones and gonad stripping yielded positive results. The research also delved into the timing and early development stages of eggs and larvae of *P. margaritifera.*

INTRODUCTION

Bivalve molluscs are an important food for most coastal inhabitants in temperate and tropical areas **(Chávez-Villalba *et al*., 2024)**. *Pinctada margaritifera* Linnaeus, 1758 (Bivalvia: Pteriidae) is known as the black-lipped pearl oyster **(Kishore & Southgate, 2016)**. This species exhibits a wide-ranging distribution in the Indo-Pacific zone, such as the Red Sea, Japan, Madagascar, Australia, and Hawaii. It also distributes on the tropical Pacific shoreline of the Americas, identified as *P. mazatlanica* as a synonym **(Tëmkin, 2010)**. *P. margaritifera,* is used to produce a type of pearl known as Tahitian cultured pearls **(El-Refi *et al.*, 2022)**. *P. margaritifera* exhibits protandric hermaphroditism, a reproductive strategy in which it undergoes a transition from the male to the female sex during its growth and development. The presence of female individuals becomes

noticeable when they reach a diameter of approximately 20 mm. However, this may vary depending on the specific habitat. **(Le Moullac *et al.*, 2012).**

Studies on *P. margaritifera* reproduction have been done around the world, from Korea, Japan and Australia **(Pouvreau *et al.*, 2000a&b; Colgan & Ponder, 2002; Le Moullac *et al.*, 2012)**; via Mexico **(Lodeiros *et al.*, 2002)**; up to Arabian Gulf **(Al-Matar *et al.*, 1993)**; in Gulf of Aden **(Aideed *et al.*, 2014)**; through the Red Sea **(Abou Zied *et al.*, 2010)**. The animals were histologically classified based on the existence of gametes in the gonads. This classification included females, males, hermaphrodites (oocytes beside spermatozoa were observed), and individuals in a sexual resting state (where no gametes were observed in the gonads, making differentiation of sex impossible) **(Mafambissa *et.al.*, 2023)**. The investigation of gonadal maturation in pearl oysters holds significant importance due to the crucial role of nucleus implantation in pearl formation, both in the context of artificial seed generation and preoperative surgeries (Karami et al., 2014). In the Red Sea, female *P. margaritifera* exhibits an annual spawning period that occurs throughout the summer and extends into the early autumn months. Also, the initiation of reproductive processes seems to be influenced by variations in sea surface temperature **(Abou Zied *et al.*, 2010; Aideed *et al.*, 2014)**.

Various methods exist for evaluating gamete development in bivalves, primarily involving the visual examination of gonads to measure their relative size, shape and colour, or the analysis of developmental phases through histological characterization. **(Assoi *et al.*, 2004)**. In addition, indices such as the gonad index **(Le Moullac *et al.*, 2009)** and mean oocyte diameter **(Kandeel *et al.*, 2013)** have been used to determine gonad grade in many studies. Histological techniques are always used to confirm reproductive events since they provide extensive information about gonad development **(Karami *et al.*, 2014)**.

According to **Nowland *et al.* (2021)**, pearl oysters can reproduce year-round, though their success heavily depends on environmental factors like water temperature and food availability. The primary physical trigger for temperate oyster species to spawn in the hatchery is a shift in water temperature. However, in tropical species, shifting salinity may be the primary trigger for reproduction. Moreover, the lack of information about species biology and larvae generation causes breeding failure **(Yigitkurt, 2021)**. Bivalve hatchery production relies on spawning induction to control reproduction and optimize fertilization. Chemical and biological stimulation and temperature shock are the main ways to initiate spawning in sexually mature bivalves. Chemical induction may be performed by injecting hydrogen peroxide, serotonin, and sex hormones into broodstock gonads or mantles. Biological stimulation conditions mature bivalves by feeding them microalgae. Additionally, gonad extract is added to the medium or broodstock's gonads. Most big clam and scallop inductions are serotonin injection. In several species of bivalves, serotonin has been shown to stimulate the spawning process successfully. **(Gibbons and Castagna, 1984; Sami *et al.*, 2021; Nowland *et al.*, 2021)**.

This study aimed to conduct an in-depth investigation into the reproductive biology, spawning pattern, and sex ratio of the *P. margaritifera* species. Additionally, the study aimed to explore the relationship between the spawning period and physicochemical characteristics. These findings are crucial for understanding the dynamics of oyster populations and can aid in large- scale artificial breeding efforts. The study also sought to explore methods for stimulating the spawning of pearl oysters within controlled hatchery environments, with the ultimate goal of facilitating large- scale oyster cultivation.

MATERIALS AND METHODS

1. Study area

Mabahiss Bay is a shallow bay in the Red Sea, Hurghada, in front of National Institute of Oceanography and Fisheries. The environmental conditions in Mabahiss Bay area are suitable for the reproduction and growth of many oysters and clams. The study area lies between latitude 27° 15′ 36″ and 27° 19′ 12″ N, and longitude 33° 45′ 00″ and 33° 49′ 48″.

2. Hydrographic parameters measurements

Hydrographic parameters include surface water salinity (S ‰), Surface water temperature and surface water pH were measured monthly in the collection site using YSI professional multiparameter. Three readings were taken for each parameter, and the mean values ± S.D. were estimated.

3. Collection and treatment of animals

Monthly sampling was conducted, from January to December 2022, either by snorkeling or SCUBA diving using hands, in depths up to 6 meters from Mabahiss Bay. Shell length of the collected animals was measured (500 animals), using vernier calipers, to the nearest 0.1 mm. The collected animals are divided into two groups; the 1st group (430 animals) was transferred to the histology laboratory for subsequent histological investigation and the 2nd group (70 animals) was transferred to the wet laboratory in NIOF, Hurghada, Red Sea branch for induce spawning experiments. The flesh parts were cautiously removed by hands and the gonads were isolated from the visceral mass carefully and fixed for 24 h in Bouin´s fixative for the histological studies.

4. Histological studies

Gonads fixed in Bouin's fixative solution were dissected and dehydrated in a series of graduated alcohol content (70, 80, 95, 100 %), cleared by passed through two consecutive baths of xylene, embedded in paraffin wax (melting point 57 - 60 °C) and sectioned into 5 µm thick slices on a fully automatic microtome (Model: ERM 4000; Hestion, Asteria). Staining with hematoxylin and eosin Y was performed on tissue sections. Gametes maturation was used to classified histological sections of each specimen.

5. Gametogenesis

The gonadal development of female and male *P. margaritifira* specimens was categorized and classified into five stages as Stage I (Early active), Stage II (Late active), Stage III (Mature), Stage IV (Spawning) and Stage V (Spent). Undifferentiated specimens will be in stage 0. The stages discussed in this study were determined based on the classification proposed by **Pouvreau *et al.* (2000a), Hwang (2007), Abou Zeid *et al.* (2010) and Mahmoud and Yassien (2023)**. These stages were identified by examining the size and density of different germ cells, which originate from gametogenesis stem cells in the gonad. Some adjustments were made to the original categorization.

6. Oocyte size-frequency

Randomly, the sections of the female gonads were scanned. Through the nucleus, fifty oocyte diameters were measured precisely at the center of the egg, where the diameter is at its maximum. This measurement was conducted using a compound microscope (Model: OPTICA B-150DB) and an Optica Vision Lite program. The oocyte diameter data were represented in the form of frequency polygons. **(Kandeel *et al.*, 2013)**.

7. Gonad Index

According to the maturity stages of *P. margaritifera*, a numerical grading approach was conducted to estimate the gonad index. Three category scores (CS), based on the development of the gonad, were explained as follows: 1 (stage I + stage V), 2 (stage II), and 3 (stage III + stage IV). The sum is obtained by multiplying the category score with the total individuals' number in each stage. The outcome is divided by the total number of specimens.

The gonad index was computed using the formula stated below by **(Yigitkurt, 2021)**:

$$GI = \frac{[n\text{stage } (I+V) \text{ x CS}] + [n\text{stage } (II) \text{ x CS}] + [n\text{stage } (III+IV) \text{ x CS}]}{\text{Total number of specimens}}$$

8. Experimental Spawning Induction

P. margaritifera broodstock were cleaned carefully by scrubbing brush and were washed with filtered seawater to remove sediment and fouling organisms. Cleaned broodstock were placed in 1.0 m^3 capacity open-flow cylindrical fiberglass conditioning tanks with a water replacement rate of 100 L/h. and held overnight under control. Continuous flow of filtered well aerated sea water was used. The following morning, broodstock were placed into a 90L plastic tank containing 1.0 µm filtered sea water to just cover the oysters to start the induce spawning experiments. Water temperature was maintained similar to the ambient temperature throughout the experiment. Several spawning induction strategies were employed on the samples, including thermal shock, gonad stripping and hormonal treatment. These methods were monthly applied from July to December 2022 to induce spawning in the *P. margaritifera* specimens as follows:

A. Thermal shock stimulation

For induced spawning by thermal shock stimulation, eight animals and two spawning tanks were utilized. In one tank, the water temperature was raised by about 10°C above the ambient temperature using water heaters, while the temperature in the other tank remained unchanged. The animals were alternately transferred between the tanks every 30 mins until spawning occurred. The thermal shock procedure was maintained for 6 hrs. Once spawning was successful, the oysters were removed from the spawning tank and placed into individual containers to complete spawning separately. Fertilized eggs were immediately collected on a 30µm nylon mesh sieve and washed briefly with filtered seawater. Eggs were incubated in moderately aerated 50L plastic tank with filtered seawater. After four hours, the fertilization process began, and within 20- 24 hours, D- stage veliger larvae appeared. These larvae were then transferred from the incubation tank to larval rearing tanks using a 30 µm nylon mesh sieve and were subsequently counted. Observations and photographs of the eggs and larvae were taken every 30 minutes.

B. Hormone stimulation

Serotonin, a neurotransmitter, induces spawning in *P. margaritifera* individuals with mature or developing gonadal tissue. If the gonad is not in a state of spawning condition, there is a possibility that the eggs may be discharged at sub-optimal phases, resulting in low egg quality and reduced survivability of the larvae. The state of gonads is influenced by both the duration and temperature. A solution containing serotonin at a concentration of 20 mM is administered through direct injection into the gonad of the oyster using a hypodermic needle with a bore size of 100 mm, which is connected to a plastic syringe. The dosage of serotonin administered is contingent upon the animal's body size. Typically, a volume of 0.5 milliliters is used for oysters of bigger size. The needle was inserted into the gonad by piercing the muscular tissue surrounding the byssal orifice. It is advisable to clean the syringe and needle with isopropyl alcohol after each oyster injection. Administering serotonin to a mature organism typically led to the stimulation of sperm release, a process that usually occurred within 2 to 5 minutes.

After the spawning process had concluded, the ova and sperm were gathered and introduced into a beaker containing 500 ml of saltwater. The ratio used was 1 ml of sperm solution to 0.5 L of water containing the ova. This mixture was allowed to incubate for a duration of 60 minutes. The fertilized eggs underwent filtration using a 40 µm nylon mesh screen to remove surplus spermatozoa. Subsequently, they were placed in 5 L tanks at a concentration of 100 eggs per ml and incubated. The temperature was maintained at a level consistent with the ambient environment. Over a period of 24 hours, a volume of 1.0 ml of water was subjected to frequent inspection and photography using a digital camera attached to a light microscope (Model: OPTICA B-150DB).

C. Gonad stripping stimulation

Female *P. margaritifera* were opened, followed by the removal of tissues and isolation of the visceral mass. The gonad was thoroughly cleaned and subsequently immersed in 1.0 µm filtered seawater. The female gonad was dissected using several incisions, facilitating the liberation of oocytes. After liberation, the oocytes underwent filtration using a 100-mm mesh to eliminate any tissue and extraneous particles. Subsequently, the oocytes were cleaned and gathered on a 30-µm mesh screen. At this juncture, the oocytes exhibited insufficient maturation for fertilization, characterized by a distinct "drop" morphology.

The process of inducing maturation in oocytes was accomplished by immersing them in a solution containing 6-mM seawater ammonia **(Ky *et al.*, 2015; Sami *et al.*, 2021)**. The oocytes were then monitored at regular intervals of 5–10 minutes until they exhibited a spherical shape, and the germinal vesicle was no longer discernible. Following a duration of approximately 50 to 60 minutes, the eggs were deemed suitable for the process of fertilization. The gonads of male oysters were processed using a method analogous to that outlined for female oysters. The male gonads were subsequently dissected using many incisions. Spermatozoa were collected within a beaker utilizing a 30-µm mesh screen to exclude any surrounding tissues.

In the process of fertilization, mature oocytes were subjected to the introduction of sperm into the designated container. Subsequently, one millilitre of the sperm solution was inserted into 0.5 litres of water containing eggs, followed by an incubation period of one hour to facilitate fertilization. The process of in vitro fertilization was conducted, followed by a subsequent step where the eggs were rinsed using filtered seawater. A 30-µm mesh screen was utilized for the purpose of removing any surplus sperm. The experiment involved placing fertilized eggs into containers with a volume of 100 liters. These containers were filled with a 1-µm filtered seawater. Seawater temperature was maintained at 25ºC to facilitate hatching.

9. Statistical analysis

The deviation from equal proportions of males and females was assessed using a Chi-square (χ^2) test with one degree of freedom. The results were displayed as means ± standard deviation (SD) and the statistical significance level was determined to be $p = 0.05$. Statistical analysis was executed using SPSS software (V 29.0).

RESULTS

1. Sex ratio

Morphologically, it is impossible to differentiate between male and female *P. margaritifera* as there are no secondary external sexual feature by which the sexes can be distinguished. However, the male gonads exhibit a milky white color, while female gonads tend to have a yellowish hue in most stages. Therefore, to determine the sex, a microscopic histological examination of gonads was performed. From January until

December 2022, 430 animals examined microscopically, the results showed that 200 animals (48.78 %) were males, 210 animals (51.22 %) were females and 20 animals were sexually undifferentiated (0.1 %) (Figs. 1, 2). A Chi-square ($\chi 2$) test with one degree of freedom revealed no significant difference in male and female proportions ($\chi 2$ value was practically zero). This suggests that *P. margaritifera* has an even sex ratio (1:1). None of the animals investigated were hermaphroditic.

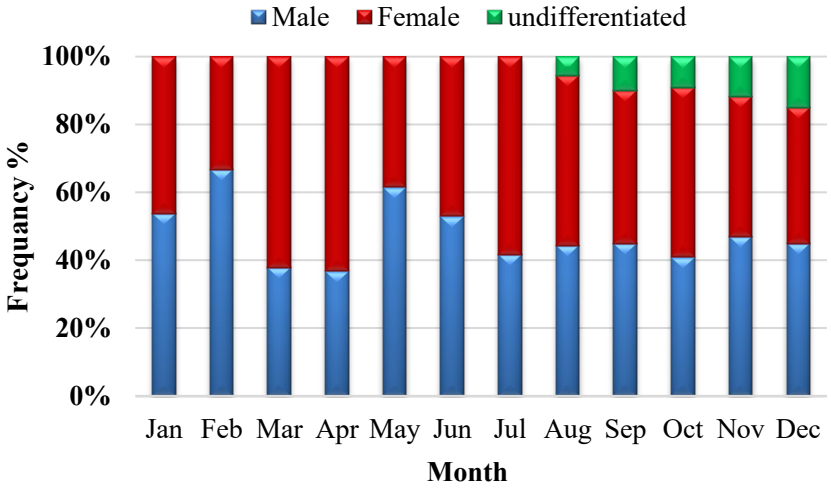

Fig. 1. Monthly Variation in sex ratio of *P. margaritifera* from Mabahiss Bay from January to December 2022

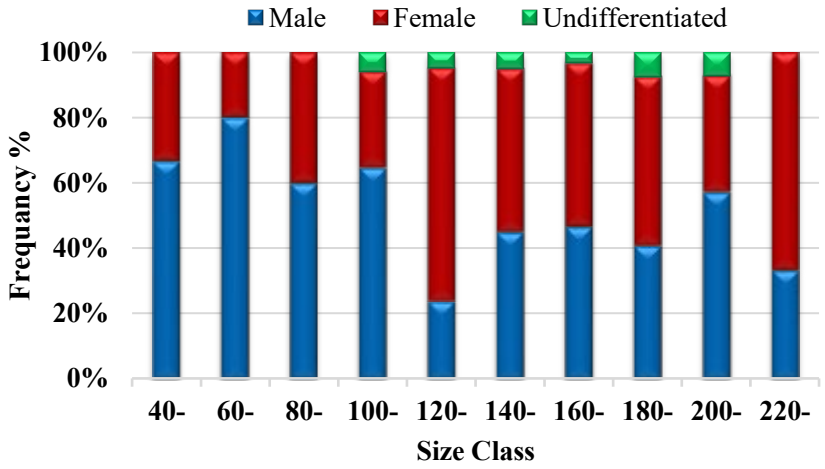

Fig. 2. Sex ratio of *P. margaritifera* according to size class from January to December 2022 in Mabahiss Bay, Red Sea

2. Gonad index (GI)

The gonad index values were computed for males, females and combined sexes of *P. margaritifera*. The results revealed monthly variation in gonad index values for both

male and female individuals. In February, the gonad index reached its minimum value of 1.4 for both sexes, indicating that all individuals were spent and inactive during this period. For males, the highest gonad index values were observed from July to August, reaching 3.0. In females, the gonad index showed peak values from April to August, ranging between 2.5 to 2.8. In combined sexes, the gonad index reached its highest values in July and August, both at 2.8 (Fig. 3). The fluctuation of gonad index for male and female indicated that there is one peak of spawning in July and August

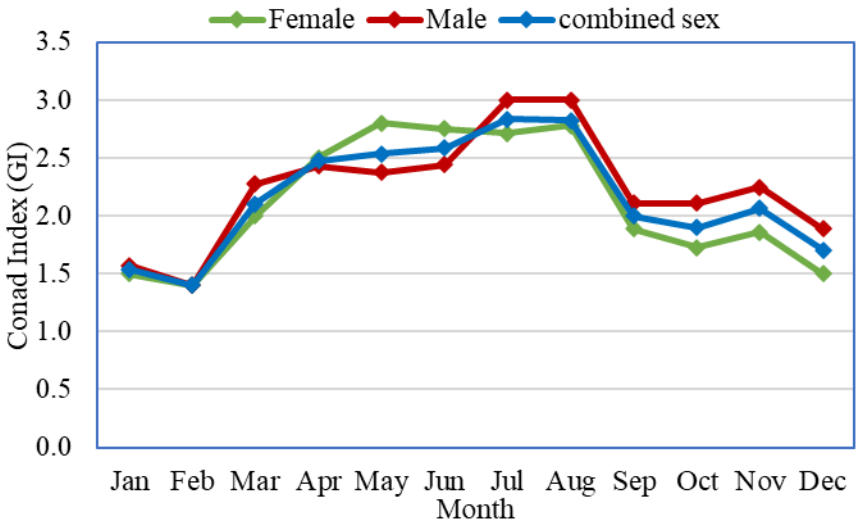

Fig. 3. Monthly variation in gonad index (GI) of female, male and combined sex of *P. margaritifera* from Mabahiss Bay from January to December 2022

3. Gametogenesis and Gonad Development

P. margaritifera sex can be determined through a histological examination of the gonads. Accordingly, the gametogenesis of the oyster in males (Fig. 4) and females (Fig. 5) can be classified into five stages besides stage 0 as shown in Table (1).

4. Gonadal cycle

The monthly development of the gonad of males and females *P. margaritifera* was illustrated in Figs. (6, 7), respectively. The pearl oyster *P. margaritifera* has one spawning period a year in summer season as the co-occurrence of different reproductive stages in all the monthly samples of the population. The presence of mature gonads in some months and the occurrence of spent stages in some months are consistent with this species having a prolonged reproductive cycle extend from Autumn to summer months.

5. Oocyte size frequency

Fig. (8) displays monthly size-frequency patterns of oocyte diameter (10 µm size class). Large percentages of small oocytes (up to 10 µm) occurred during January and

February indicating oogenesis beginning. The relative frequency of small oocytes was clearly absent in October, November and December.

Table 1. Histological description of male and female *P. margaritifera* maturity stages

Maturity Stages	Male	Female
Stage (0) Inactive Stage	Sex cannot be identified, and the gonad is actionless or inactive after spawning (resting). In this degenerative stage, only connective tissues are detected, while the follicles exhibit a collapsed state and reduced volume (Fig. 4F).	
Stage (I) Early active stage (EAS)	Follicle walls and stem cells lining them developed, gametes proliferated and rounded to enlarged follicles filled with spermatogonia and few spermatocytes (Fig. 5A).	Small primary oogonia with a rounded large nucleus, and thick cytoplasm, which develops into oocytes attached to the follicle wall and have prominent chromatin nuclei. Free oocytes are absent in the lumen (Fig. 4A).
Stage (II) Late active stage (LAS)	The presence of spermatogonia, spermatocytes, spermatids, and spermatozoa are notable inside the follicles. Although observing spermatids and spermatozoa is possible, it is limited to tiny quantities and requires high magnification. (Fig. 5B).	Follicles' lumens are filled with more developed and appear like pear-shaped oocytes, attached to the follicle wall through the peduncle. They have an apparent nucleus and nucleolus. Free oocytes make up less than half of the follicles' components; attached oocytes are plentiful, while oogonia are rare (Fig. 4B).
Stage (III) Mature Stage (MS)	Large numbers of developed spermatozoa pointing towards the center produce concentric rings or plugs in the follicles. Very ripe specimens have tidy follicles with spermatozoa bands close to the wall. The visual presentation of follicles is orderly (Fig. 5C).	Follicles swell, take up more space, and fuse. The lumen of the follicles is filled with many mature, round, and expanded oocytes; the follicles have a polygonal shape; the walls of the follicles are swollen and indefinite; and the lining is made up of thin epithelium layers. (Fig. 4C).
Stage (IV) Partially spawning Stage (PSS)	Spermatozoa are clearly visible in a swirling shape. Some follicles have space due to the release of mature spermatozoa (Fig. 5D).	Certain follicles become vacant due to the liberation of unfertilized oocytes, leading to the rupture of the follicular walls. (Fig. 4D).
Stage (V) Spent Stage (SS)	Residual spermatozoa undergoing resorption are found in the lumen. Phagocytes are present (Fig. 5E).	The broken follicles appear relatively empty and scattered, and some residual oocytes undergoing resorption are found in the follicles. Phagocytes are present (Fig. 4E).

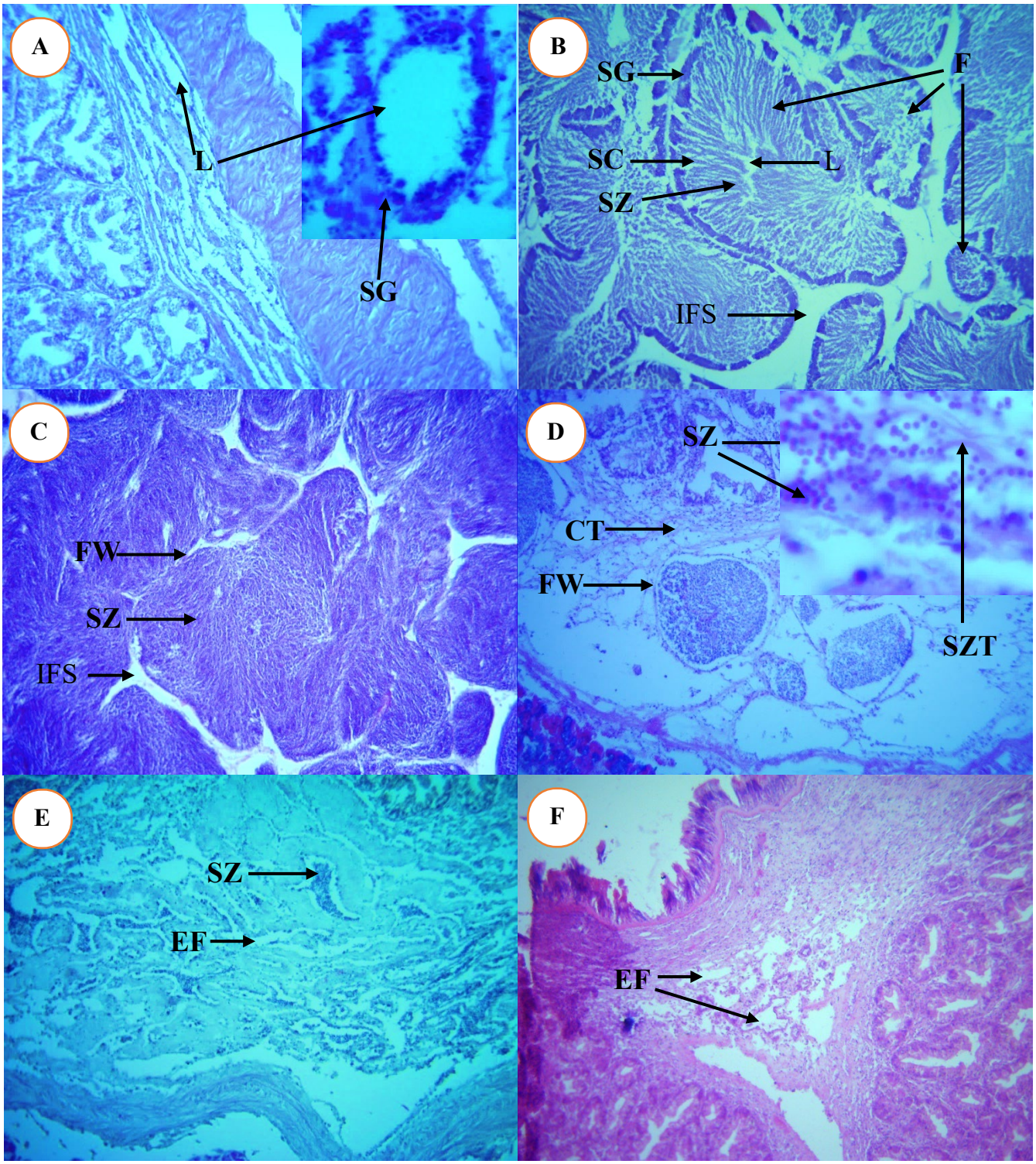

Fig. 4. Photomicrograph of Gonadal development stages of male *Pinctada margaritifera*
A: early active stage; B: late active stage; C: ripe stage; D: partially spent stage; E: spent stage; F: Stage 0 (inactive stage); CT: connective tissue; EF: empty follicle; F: follicle; FW: follicle wall; IFS: interfollicular space; L: lumen; SC: spermatocyte; SG: spermatogonia; SZ: spermatozoa; SZT: spermatozoa tail. (100x) enlarged portion of A in left panel (400X).

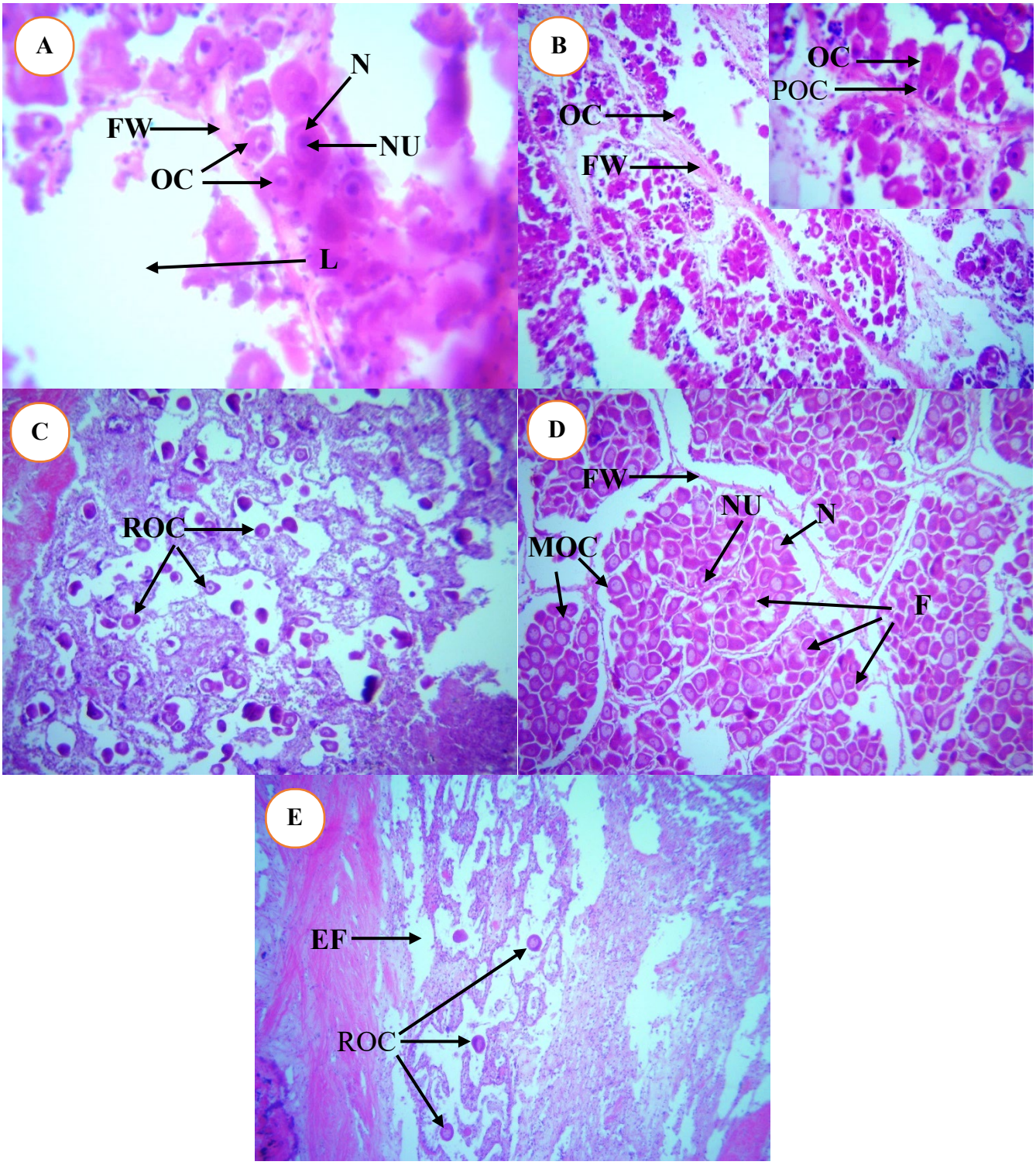

Fig. 5. Photomicrograph of Gonadal development stages of female *Pinctada margaritifera*

A: early active stage; B: late active stage; C: ripe stage; D: partially spent stage; E: spent stage; CT: connective tissue; EF: empty follicle; F: follicle; FW: follicle wall; IFS: interfollicular space; L: lumen; MOC: mature oocyte; N: nucleus; NU; nucleolus; OC: mature oocyte; POC: peduncle of oocyte; ROC: residual oocyte. (100x), enlarged portion of B in right panel (400X).

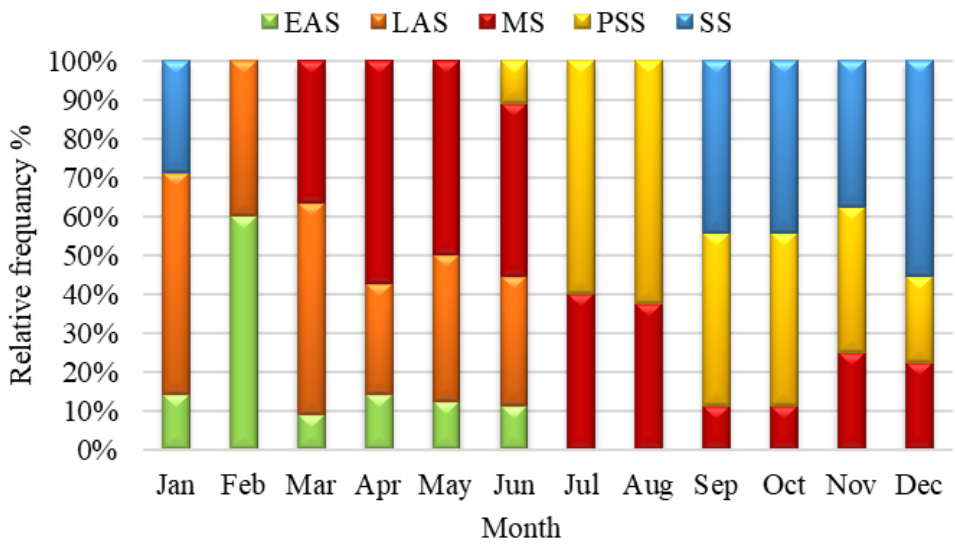

Fig. 6. Monthly variation in the relative frequency of different spermatogenesis stages of male *P. margaritifera* from Mabahiss Bay from January to December 2022.

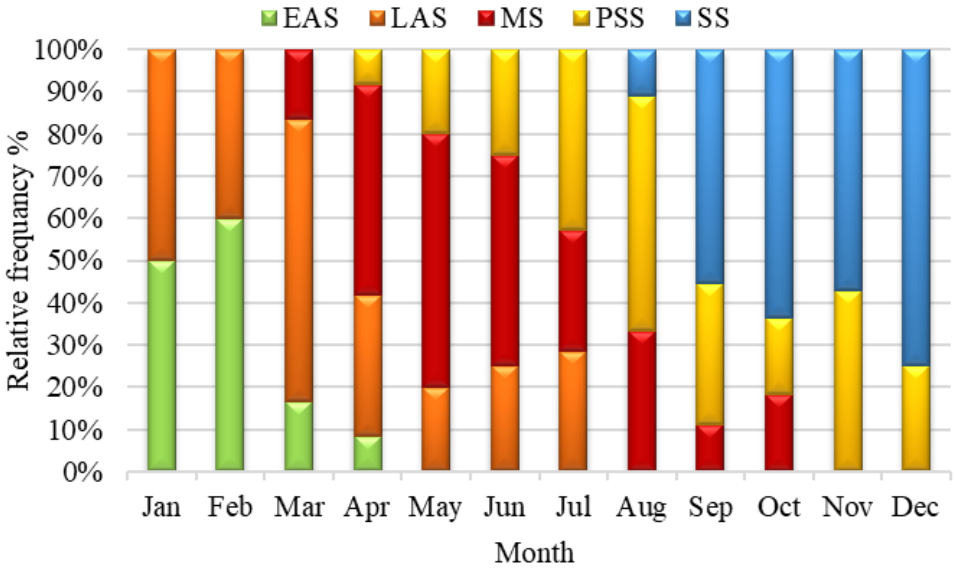

Fig.7. Monthly variation in the relative frequency of different spermatogenesis stages of female *P. margaritifera* from Mabahiss Bay from January to December 2022.

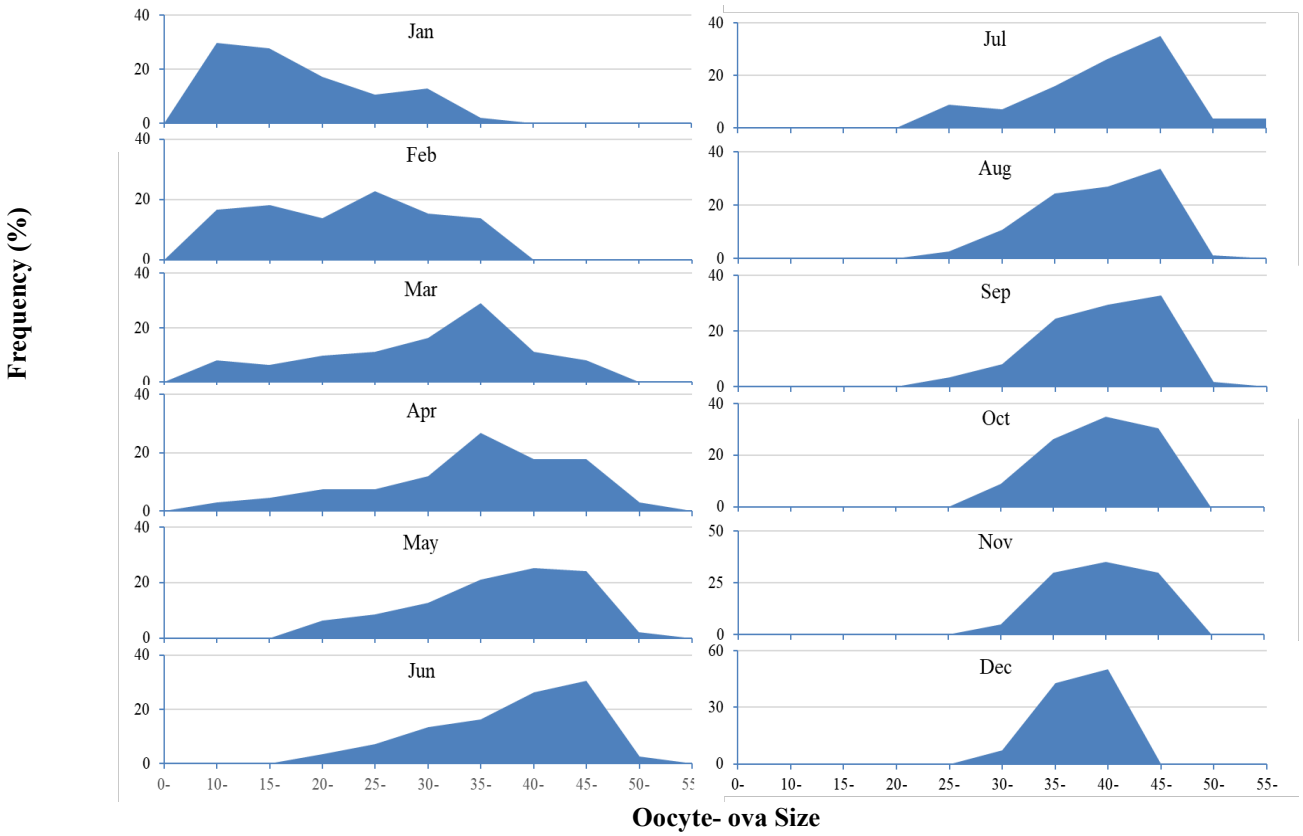

Fig. 8. Relative distribution of oocyte-ova diameter of various size classes in female gonads of Pinctada margaritifera from January to December 2022 from Mabahiss Bay (oocyte-ova size grouped in 10 µm size classes).

6. Induced spawning

A total of 70 animals were selected and prepared for the purpose of inducing spawning throughout the study period. Each month eight animals were tested to induce spawning by hormones, thermal shock, and gonad stripping. Of the three techniques used to stimulate spawning of oysters, only two had positive outcomes: hormones and gonad stripping. Timing and size of the early development of egg and larvae of *P. margaritifera* illustrated in Table 2.

Table 2. Timing and size of the early development of *Pinctada margaritifera.*

Stage	Size µm	Time after induce spawning
Fertilization	45-55	15-30 min
Zygote with polar body	50-55	30-60 min
1st cleavage	50-55	60-90 min
Morula	55-65	4-8 hrs.
Trochophore	60-70	7-15 hrs.
D-shape larvae	65-80	20-24 hrs.

After female *P. margaritifera* gonad striping, the oocytes were not sufficiently mature for fecundation, immature oocytes appeared in "drop" shape (Fig. 9A). Artificial maturation of the oocytes was shown by the presence of a germinal vesicle after 30-50 min in seawater ammonia, and they had attained a round shape (Fig. 9B). After approximately 60 minutes, the oocytes displayed absence of the germinal vesicle and had an assumed rounded shape, indicating their readiness for fertilization.

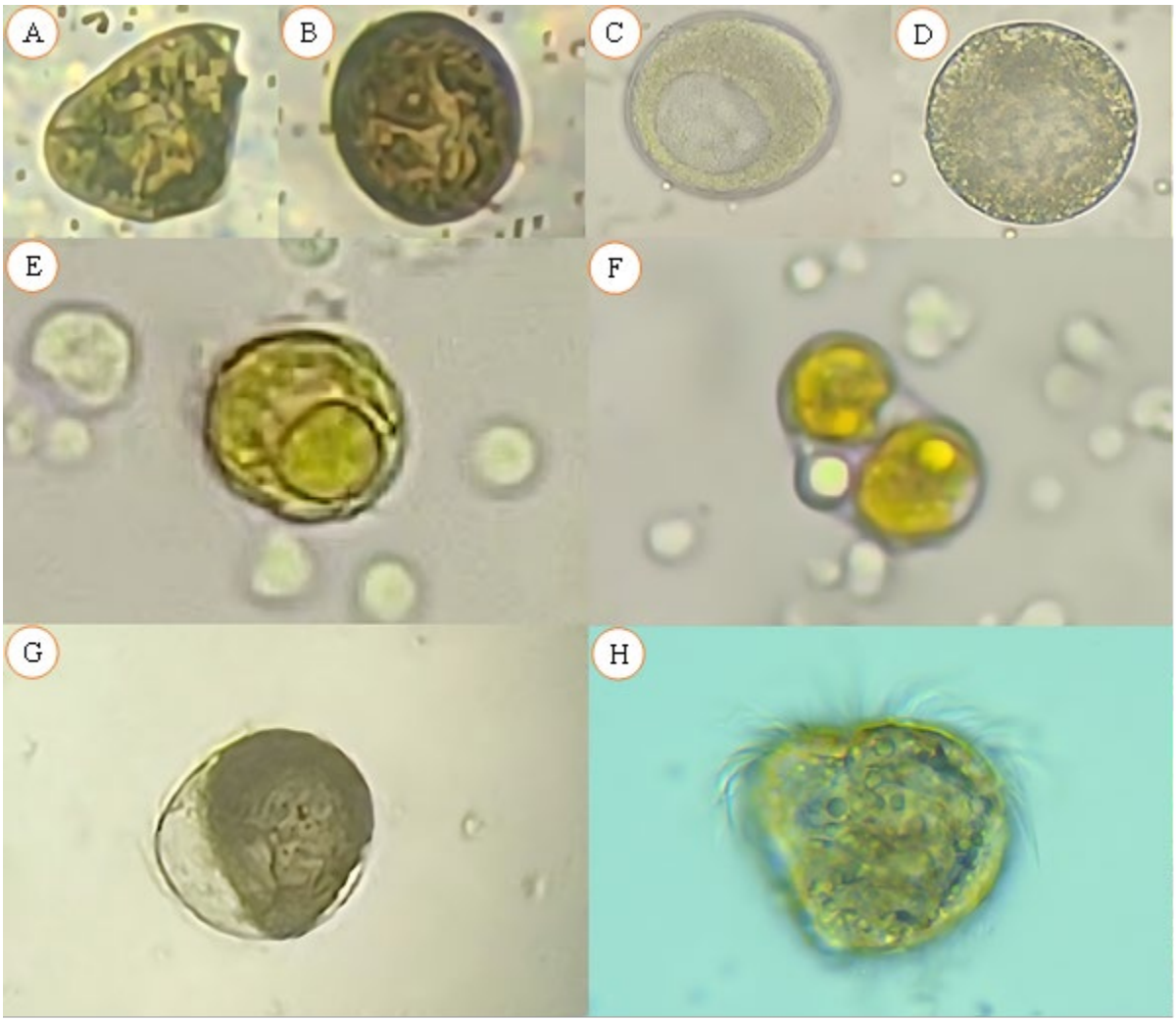

Fig. 9. Showing egg and larvae development of *Pinctada margaritifera.*

A: Unfertilized droplet oocyte after releasing by gonad stripped; B: Unfertilized mature oocyte; C: Unfertilized mature oocyte after releasing by hormone induce spawning; D: Fertilized oocyte 15-30 min after releasing by hormone induce spawning; E: Zygote shows polar body 30-60 min after releasing; F: 1st cleavage 60-90 min after releasing; G: Immobile morula after 6-9 hrs. of fertilization; H: Trochophore larvae 15 hrs. after fertilization; I: D-shaped larva with ciliated velum 20 hrs. after fertilization

Serotonin injection into a mature organism often results in the prompt initiation of sperm release, typically occurring within 2 to 5 minutes. Following the hormone- induced spawning of female *P. margaritifera*, the oocytes were immediately observed,

characterized by the presence of a germinal vesicle (Fig. 9C). These oocytes were spherical and measured between 45- 55µm in diameter. After 15- 60min, the oocyte was ready for fertilization (Fig. 9D), and then the first polar body appeared (Fig. 9E). The first cleavage event, namely the formation of two cells, was seen around 30 minutes after the release of the first polar body, with a size of 50- 55µm (Fig. 9F). The trochophore larvae (Fig. 9G) appeared during 7hrs with a size of 60- 70µm. The first D- shaped veliger, measuring 65- 80µm, became observable around 20hrs after fertilization (Fig. 9H).

DISCUSSION

The pearl oyster *P. margaritifera* exhibits protandric hermaphroditism, whereby individuals begin their life cycle as males and then transition to females over many years. This transition process occurs gradually, with the sex ratio in populations older than eight years and with a shell height above 180 mm approaching a balanced proportion of males to females, around 1:1. **(Chávez-Villalba *et al.*, 2011)**. The present study agreed with the previous observation and found that the proportion of males and females equals 1:1.05, which points to the fact that the sex ratio in *P. margaritifera* is even (1:1), did not differ from the observations made by **Mahmoud (2003)** he stated that sex ratios are usually close to 1:1 in bivalve populations. **Pouvreau *et al.* (2000a)** observed that 2-year-old oysters sex ratio was 0.1:1.0 for females and males, respectively and **Kimani and Mavuti (2002)** in Gazi Bay, Kenya, showed that females appear at about 2-year-old oysters, 100-110 mm height. These results disagreed with the findings of **Aideed *et al.* (2014)**, who showed that the percentage of female *P margaritifera* was one hundred percent in shell height of more than 130 mm. From the previous studies of oysters, it has been observed that the proportion of male to female individuals is contingent upon the dimensions of their shells. An inverse relationship exists between shell sizes and the sex distribution among individuals. Specifically, as shell sizes decrease, the percentage of males increases; conversely, the proportion of females increases as shell sizes increase. The ratio becomes equal if all sizes particularly the small sizes (less than 40 mm) are represented, as in the present study (40-240 mm).

Chávez-Villalba *et al.* (2011) stated that There was a lack of empirical information about the impact of biotic & abiotic parameters, as well as other variables, on sex development during the first two years of an individual's existence. Several factors, including temperature changes, food availability, and stress, appear to have an impact on specimens older than two years, particularly females. **Kimani and Mavuti (2002)** in Gazi Bay, Kenya, showed that the undifferentiated oysters *P. margaritifera* occurred only in specimens more than 130 mm. In the present study, females appeared in specimens more than 40 mm. and the undifferentiated specimens occurred only in pearl oysters more than 100 mm. It seems that sex changes may be happened swiftly; there are few undifferentiated pearl oysters in the sampled community. In the present study, there weren't found hermaphroditic individuals among the oyster examined; it agreed with the

observations made by **Chávez-Villalba *et al.* (2011),** who showed that only one hermaphrodite from the 1067 pearl oysters that were examined, and **Kimani and Mavuti (2002)**, who found only three hermaphrodites of all the 331 oysters examined.

The gonad index is the quantitative measure that is used most often when attempting to determine the level of reproductive activity in marine bivalves. Many authors expressed the gonad index by calculating the ratio between gonad weight and flesh weight **(Kandeel *et al.,* 2013; Aideed *et al.,* 2014)**. The current research used a numerical grading approach to estimate the gonad index, which was determined by assessing the maturity stages of pearl oysters, based on the gonadal development according to the formula presented by **(Yigitkurt, 2021)**. The high values of the gonad index were synchronized with the maturity of the gonad, while the lower values seen after the high values were an indicator of spawning **(Sami *et al.,* 2021).** Monthly variations of gonad index values of males and females followed virtually the same pattern, and the gonad index values for *P. margaritifera* confirmed the behaviour of maturity phases. When the male and female gonad indexes were considered together, one clear peak appears in the autumn and summer.

Yigitkurt (2021) observed similar findings in *P. imbricata* radiata specimens located in Izmir Bay, where these individuals attained their first reproductive maturity during April. The observation of spawning activity occurred throughout the summer period from June to September, coinciding with months of peak of the gonad index levels. **Aideed *et al.* (2014)** found that there are two apparent peaks and an obvious decline in the gonad index. The two peaks are probably parallel to a long phase of high maturity stages between January- November. In the present study, a significant positive association exists between temperature and the gonad index. The highest gonad index was recorded in August (28.65°C), whereas the lowest one was recorded in February (22.3°C).

Gonad development refers to the dynamic alterations that take place in the gonad during both the active and inactive reproductive phases. Several methods exist for evaluating gamete development in bivalves such as the shape, size and color of the gonads or histological description, in addition to the use of indices such as the mean gonad index for population. Histological methods are usually employed to validate reproductive events since they give substantial gonad development information **(Karami *et al.,* 2014)**. *P. margaritifera* reproduction was previously studied by **Abou Zied *et al.* (2010)** in the Red Sea. The present study's findings agree with those authors and show that *P. margaritifera* has one spawning period a year in the summer season.

The confirmation of the annual spawning pattern was achieved by the identification of polymodal monthly distribution in the size- frequency of oocyte- ova throughout the year. The results showed that the large percentages of small oocytes (10µm) occurred during January and February, pointing to the start of oogenesis. A significant increase in the relative frequency of large oocytes with a size of 35µm was

observed in March to April, followed by a shift to a size of 45µm from May to November. This indicates that the beginning of sexual activity and gamete regeneration is in January and February, and that the peak of sexual maturity and spawning occurs in the summer months, especially August.

Pearl oysters, like most bivalve species, undergo a phase of planktonic larvae that can disperse, which is subject to the effect of biological and physical factors. The efficacy of the larval stage is contingent upon a diverse range of characteristics and processes, including but not limited to temperature, salinity, food availability, and predation **(Thomas *et al.*, 2011)**. In the present study, variations of physicochemical parameters such as surface water temperature, pH and salinity were monthly recorded in Mabahiss Bay, Hurghada, Red Sea (Fig. 10), and their values coincide with those of **Farah (1982)**, who reported that 19 to 35ºC sea water temperatures and 41 to 45‰ salinity is suitable for oyster culture. **Elamin *et al.* (2020)** stated that July is considered the onset of the spawning season of oysters in the Red Sea (33ºC).

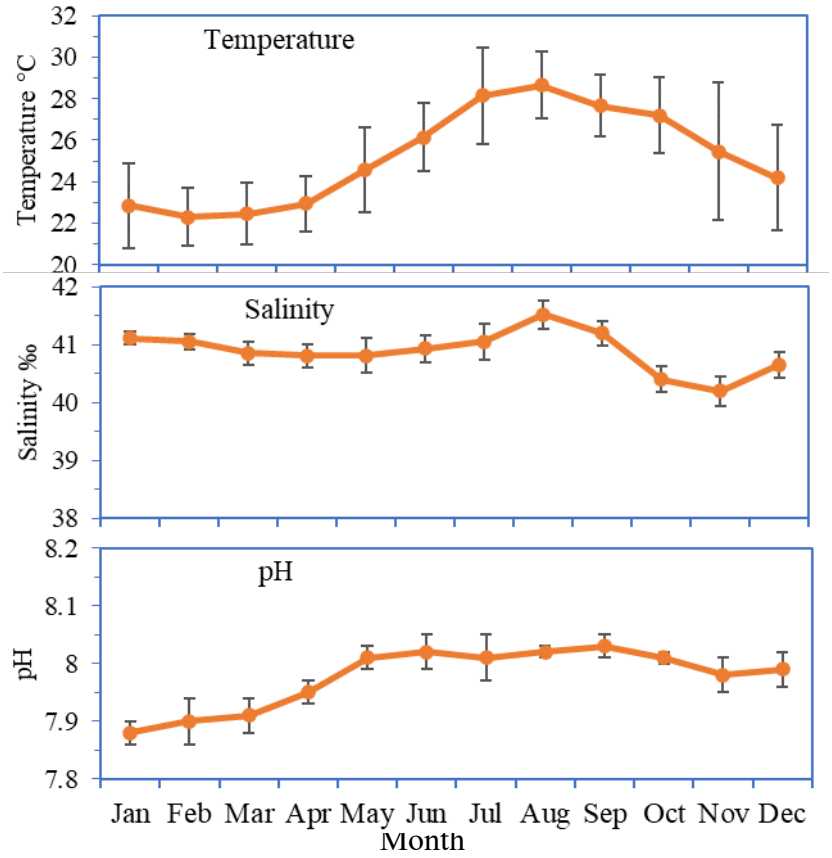

Fig. 10. Monthly physicochemical parameters (± SD) in Mabahiss bay, Hurghada, the Red Sea during the period from January to December 2022.

According to **Mulyana *et al.* (2018)**, the developmental stage and larval size in *P. margaritifera* exhibit similarities to those reported in *P. fucata* and *P. maxima*. The pearl

oyster exhibits a scattered larval phase throughout its life cycle. According to Rose and **Baker (1994)**, a fertilized egg's developmental process typically spans 12 to 24 days. The larval development of *P. Margaritifera* has been documented in studies conducted by **Alagarswami *et al.* (1989)** and **Doroudi and Southgate (2003)**. The present study showed that in case of serotonin stimulation method the mature oocyte has been ready for fertilization 30 min after hatching but in case of gonad stripped method the mature oocyte has been ready for fertilization 60 min after hatching.

The first polar body appeared approximately around 24 minutes after the fertilization process, whereas the fertilized ova exhibited an average diameter ranging from 45 to 55 µm. After 24 hours, during which the larvae reached the D-stage characterized by a D-shaped shell, they were gathered using a 25 µm mesh filter and placed in aerated fiberglass tanks with a volume of 90 liters. The larvae were stocked at a density of 2 individuals per millilitre.

The effective management of *P. margaritifera* production necessitates the capability to anticipate the spots that are most likely to be inhabited by larvae, hence enabling the focused selection of spit collecting regions. The achievement of this objective may be attained by the monitoring of larval presence in samples of plankton. Therefore, it is important to get species-specific data about the distribution of larvae for the targeted species in order to comprehensively comprehend the fluctuations in recruitment. Pearl oysters, similar to the majority of bivalve species, undergo a phase of planktonic larval development, during which their dispersion capabilities are impacted by several biological and physical factors. The larval phase's success is contingent upon a diverse range of characteristics and processes, including temperature, salinity, food availability, and predation **(Thomas *et al.*, 2011)**.

CONCLUSION

A comprehensive understanding of reproductive biology of *Pinctada margaritifera* is essential to developing sustainable methods for the long-term utilization of this valuable resource. There is an inverse relationship exists between shell sizes and the sex distribution among individuals. The investigation of reproductive patterns in this species has revealed a distinct spawning phase extending from June to October, with a notable peak occurring in July and August. Of the three techniques used to stimulate spawning in oysters, only two had positive outcomes: hormones and gonad stripping.

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